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Effect of acid Lugol solution as preservative on two representative chitineous and gelatinous zooplankton groups

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Abstract

The estimation of biomass from body lengths to carbon regressions is a common approach in plankton research. Several different chemicals for sample preservation are in use, and conversion factors to account for shrinkage effects exist, but to our knowledge the consequences of using potassium-iodide and iodine (Lugol solution) as preservative on body sizes of different mesozooplankton groups have not been investigated. We tested the effect of 2% acidified Lugol solution on body sizes over time on two major marine mesozooplankton groups, namely larvaceans and copepods, which are representatives of gelatinous and chitineous plankton, respectively. For both, we observed a significant shrinkage effect over time ($P < 0.0001$). Larvaceans showed a reduction in body size by 20% within the first 2 min, which stabilized after 36 h at 22%, whereas copepods shrank by 17%. These differences were significant ($P = 0.0324$), with no further shrinkage observed over an additional 3 months. Failure to adequately account for shrinkage effects could result in significant biomass underestimation if length–weight relationships are generated from live material.

Introduction

It is common practice in limnology and oceanography to estimate biomass and evaluate carbon fluxes based on abundance and size measurements of different plankton groups (Vargas et al. 2007). Fixation and preservation of samples are generally necessary for practical enumeration and biovolume estimation in subsequent laboratory analysis. Several chemicals are in use, and traditionally aldehyde-based solutions, like glutaraldehyde or buffered formaldehyde, have been used particularly for marine mesozooplankton samples. Less toxic iodine-based alternatives are primarily used for protozooplankton (Throndsen 1978) and freshwater samples of phyto- and zooplankton (e.g., Goldyn and Kowalczewska-Madura 2008, Okun et al. 2008). Most integrated marine plankton community studies with parallel investigation of microbial and mesozooplankton components have been fixed and preserved with Lugol solution and formaldehyde, respectively. These chemicals influence osmotic balance and lead to a change in cell volumes (Ohman and Snyder 1991). Length to dry weight/carbon conversions are often based on live animals (Berggreen et al. 1988), and sizes of preserved samples then need to be recalibrated to account for size changes due to fixation.

In general, formalin is reported to show lower cell shrinkage effects than Lugol on protozooplankton (Choi and Stoecker 1989, Ohman and Snyder 1991, Stoecker et al. 1994, Karayanni et al. 2004), whereas no differences were found for larvacean trunk lengths fixed and preserved with 5% neutralized formaldehyde and acidified Lugol solution (Nakamura et al. 1997). The effect of formaldehyde has been examined for larvaceans and crustaceans, with no reported shrinkage effect for the latter (Black and Dodson 2003, Põllupüü 2007). Shrinkage rates on body lengths of larvaceans due to formalin fixation range from 3.4% to 30.6% (Alldredge 1981, Hopcroft and Roff 1998) but are typically reported to be 10% to 13% (Deibel 1988, Landry et al. 1994, Scheinberg et al. 2005). The effect of Lugol solution on copepod sizes has not been investigated. Most Lugol investigations focused on protozoans and showed a decline in cell volume by 25% to 57%, with a reduction in total length of 3% to 10% (Putt and Stoecker 1989, Montagnes et al. 1994, Menden-Deuer et al. 2001).

Numerous studies measuring mesozooplankton have used Lugol solution for larvaceans (Knoechel and Steelflynn 1989, Nakamura et al. 1997, Broms and Tiselius 2003, Koski et al. 2007) and crustacean plankton in fresh water (Brett et al.

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1994, Wiackowski et al. 1994, Kozak and Goldyn 2004, Gyllström et al. 2005, Hansson et al. 2007, Goldyn and Kowalczewska-Madura 2008, Okun et al. 2008) as well as marine environments or laboratory experiments (Schmidt et al. 2002, Arendt et al. 2005, Koski et al. 2005, Poulsen and Kiørboe 2006, Koski 2007, Safi et al. 2007, Köster et al. 2008). However, none of the studies reported and considered shrinkage despite such documented impact.

The objective of this study is to evaluate the effect of 2% acidified Lugol solution on two major mesozooplankters, larvaceans and copepods. We repeated measurements of individuals from fixation up to 3 months of preservation and derived shrinkage rates which should be taken into account when estimating zooplankton biomass.

Materials and methods

Shrinkage rates due to fixation and preservation of samples with 2% acidified Lugol solution were estimated for the time intervals 2 min (larvaceans only), 36 h, and 3 months after addition of Lugol solution. Samples were collected on June 5, 2007 in the northern end of the Aarhus Bight, southern Kattegat, Denmark. Observed salinities at the sampling site ranged from 15.7 at the surface to 27.1 at the bottom (Miljøcenter Aarhus, unpublished data). Mesozooplankton was sampled using a 90-µm WP2 net with a nonfiltering cod-end and hand tows from approximately 12 m to the surface. Samples from several tows at the same position were stored in an insulated box and immediately transferred to the laboratory for further analysis. Size measurements were carried out under a binocular microscope with an ocular micrometer at a magnification of ×120 and ×250, measured to the nearest 20 and 10 µm, respectively. Specimens were sorted out individually with a pipette (larvaceans with a wide-bored plastic pipette) and transferred to one cavity each within a 24-well tissue culture tray (NUNCTM multiwells) and diluted with 20 µm filtered seawater.

All live measurements were conducted in triplicate before direct addition of Lugol to a final concentration of about 2% (Menden-Deuer et al. 2001). To evaluate the shrinkage effect over time, the same specimen was measured 2 min (larvaceans only, triplicate measurements), 36 h (single measurement), and 3 months (single measurement) after treatment. Trunk length and tail length were measured on larvaceans, whereas cephalothorax and total body lengths were used as the size measure for copepod and copepod nauplii, respectively. For length measurements of curled-up larvacean tails, the eyepiece with the ocular micrometer or the sample itself was rotated for stepwise measurements or the tail was straightened out with dissecting needles if possible. Neither group was identified to finer taxonomic levels. Samples were stored at 15°C in darkness and inspected regularly.

In statistical analysis, the distributions of the size measurements were first examined for larvacean trunk and tail lengths, copepod and copepod nauplii sizes with respect to time after preservation by repeated measures analysis. Size measure (s) was analyzed in a hierarchical analysis with difference between larvaceans and copepods (group, g) and time after preservation (t) as main effects. The difference between tail and trunk length for larvaceans, as well as the difference between adults and nauplii for copepods, was a nested fixed effect (measure, m) within group. The interactions between time and group, as well as the interaction between time and measure, were modeled as fixed effects, whereas differences between individuals (I) was a random effect specific to group and measure. In summary, following is the model for the log-transformed response variable:

$$\log(s_{ijkl}) = g_i + m_{j(i)} + t_k + (t_k \times g_i) + [t_k \times m_{j(i)}] + I_{l(i)} + e_{ijkl} \qquad (1)$$

where i, j, k, and l are indexes for the levels of group, measure, time, and individuals, respectively, and e_{ijkl} describes the residual variation.

Thus, the random variation between individuals [$I_{l(i)}$] was described by means of four different distributions, i.e., the size measures on individuals followed different distributions depending on whether it was the larvacean trunk or tail or the length of copepod or copepod nauplii that was measured. Thus, the model is a repeated measures analysis where differences in size measurements between individuals before preservation were accounted for by the stochastic factor $I_{l(i)}$. The change in size from one time point to the next was investigated for both larvaceans and copepods by formulating contrasts of the model. The analysis was carried out using the statistical software SAS (PROC MIXED).

Assessment

We analyzed 132 animals, distributed over 20 larvaceans, 59 copepods, and 53 nauplii, for the four different time periods, resulting in 452 observations used in the analysis (triplicate measurements on live animals and larvaceans 2 min after Lugol addition were averaged to produce one observation). The number of observations for each time period was almost identical for live animals (144 observations) and 36 h and 3 months after addition of Lugol solution (143 observations, 1 larvacean specimen lost after the 2-min measurement, destroyed due to handling), whereas 20 measurements on larvaceans only were carried out 2 min after addition of Lugol solution. The average trunk and tail size measures decreased by 20%–23% after treatment, whereas copepods and copepod nauplii lengths were reduced by about 16.5% (Fig. 1).

All effects of the full model in Eq. 1 were significant except for the interaction of time and measure ($t_k \times m_{j(i)}$) ($F_{5,298} = 1.10$, $P = 0.3594$). Thus, there was no difference in shrinkage with time between larvacean trunk and tail, and between copepods and their nauplii. Consequently, this effect was removed and the model reanalyzed. There was a significant difference in the size measures between the two groups and between trunk versus tail and copepod versus nauplii within these two groups (Table 1). There was also a significant general difference in size

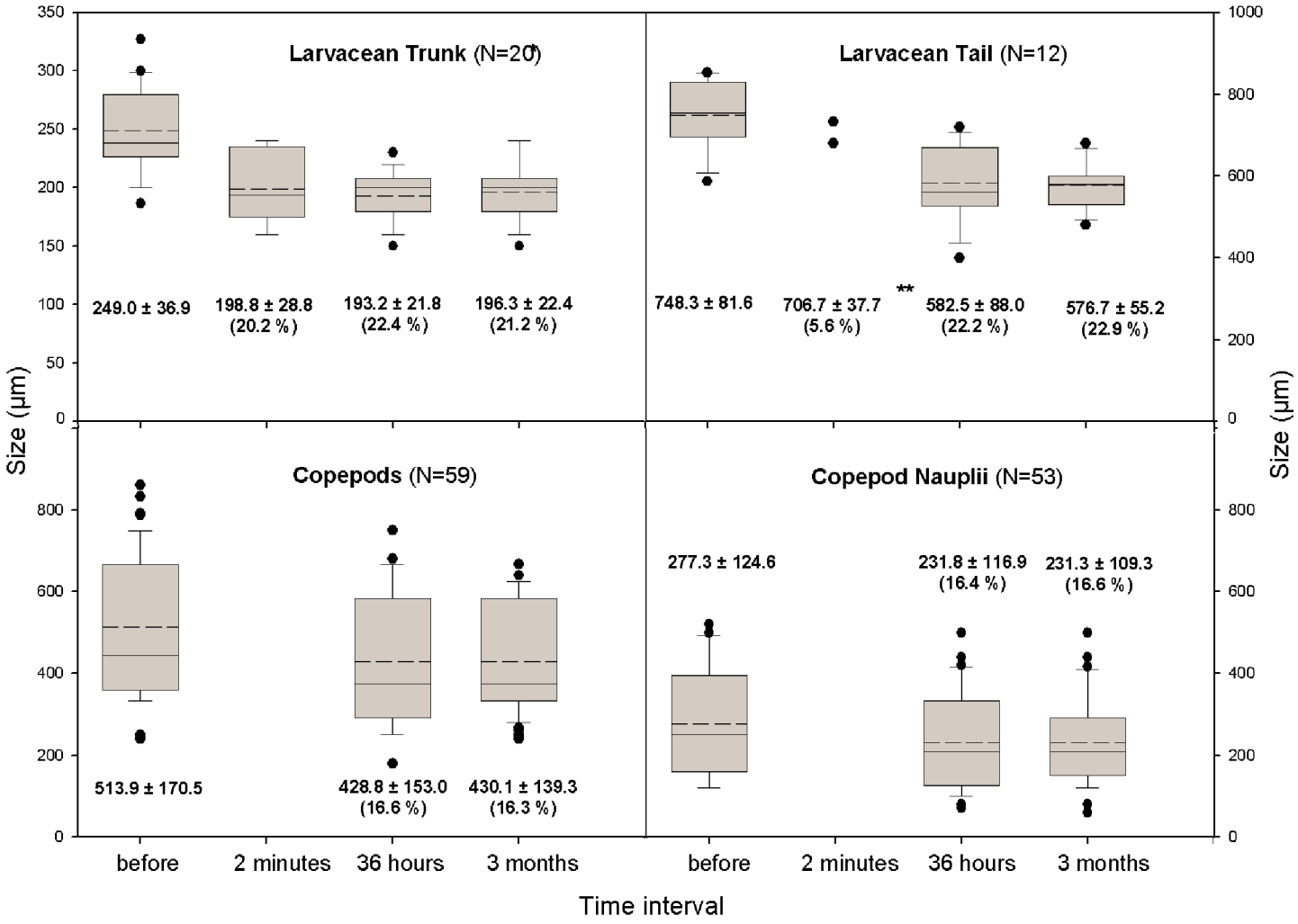

Fig. 1. Box-whisker plot of different size measures (larvacean trunk and tail, copepods including copepodites and copepod nauplii) before and three separate times after addition of acidified Lugol solution, 2% final concentration. Median indicated by solid line within the box, dashed line presents the overall mean, 75th and 25th percentiles stated in box; 90th and 10th percentiles indicated by error bars. Mean overall sizes and SD as well as reduction relative to mean live sizes are indicated for each box. *n = 19 for 36-h and 3-month intervals; **based on two individuals only.

measures between times of analysis, and this difference was specific to larvaceans and copepods, although $t_k \times g_i$ was not as significant as the other fixed effects of the model. The random variation between nauplii individuals was the largest (± 49%), followed by copepods (± 33%), whereas variation between larvacean trunk and tail sizes (± 9%–11%) were of the same magnitude as the unexplained residual variation.

For both larvaceans and copepods, the overall significant shrinkage with time was due to a large size reduction shortly after addition of the Lugol solution, whereas there was no further significant shrinkage from 36 h to 3 months after initial treatment (Table 1). In fact, for larvacean size measures that were also measured 2 min later, the shrinkage almost occurred instantaneously after Lugol addition, with a smaller, however not significant, size reduction from 2 min to 36 h (Table 1). There was no indication of changes in size measures from 36 h to 3 months for larvaceans or copepods. The average shrinkage rate after 36 h and 3 months, calculated from expected values of the model, were 22.34% and 17.49% for larvaceans and copepods, respectively.

Discussion

In this study we show that the use of 2% acidified Lugol solution for fixation and preservation of mesozooplankton samples leads to a significant shrinkage effect on sizes for larvaceans and copepods. This has also been demonstrated in previous studies for ciliates (Putt and Stoecker 1989, Ohman and Snyder 1991, Jerome et al. 1993, Stoecker et al. 1994), flagellates (Borsheim and Bratbak 1987, Choi and Stoecker 1989, Menden-Deuer et al. 2001), several phytoplankton groups

Table 1. Test of effects (*F* test) and contrasts (*t* test) in model for the log-transformed size measures.

Sources of variation	Degrees of freedom (df)	*F*/*t* value	*P*
Effects			
Group	1, 303	13.75	0.0002
Measure	2, 303	435.06	<0.0001
Time	3, 303	125.77	<0.0001
Group × time	2, 303	3.47	0.0324
Contrasts of group × time			
Larvaceans			
$Time_0$ versus $time_1$	303	7.93	<0.0001
$Time_1$ versus $time_2$	303	1.73	0.0853
$Time_2$ versus $time_3$	303	–0.37	0.7115
Copepods			
$Time_0$ versus $time_2$	303	15.99	<0.0001
$Time_2$ versus $time_3$	303	–1.14	0.2568

For copepods, there were no contrasts involving $time_1$, since no copepods were measured 2 min after treatment (addition of acidified Lugol solution). $Time_0$, live animals; $time_1$, 2 min after treatment; $time_2$, 36 h after treatment; $time_3$, 3 months after treatment.

(Verity et al. 1992, Montagnes et al. 1994), and cyanobacteria (Hawkins et al. 2005), with an average protozoan volume reduction of 33% to 40%. Maximum shrinkage rates are published, with a 40% volume reduction for cyanobacteria (Hawkins et al. 2005), 57% for naked heterotrophic flagellates (Borsheim and Bratbak 1987), and 45% for ciliates (Jerome et al. 1993). Studies of seven different phytoplankton groups showed a mean length reduction by 10.7% (ranging from 3.1% to 22.2%), including three Bacillariophyceae species (mean size reduction 7.1%) (Montagnes et al. 1994). This shows that in the case of Bacillariophyceae, the existence of a silicate frustel does not prevent size reduction due to the use of acidified Lugol solution (Montagnes et al. 1994). Our results confirm this, as we show that irrespective of existence of an exoskeleton in the case of copepods, shrinkage occurs due to the use of acidified Lugol solution but to a higher degree than found for Bacillariophyceae. Comparing different mesozooplankton groups, the composition of the matrix structure seems essential to the degree of size reduction, with gelatinous larvaceans showing a significantly higher shrinkage rate than copepods and their nauplii.

Assuming that biomass is related to body size by an exponent of 2 or 3, larvacean biomass is underestimated by 40% and 53%, whereas copepods' biomass would be biased by 31% and 43%. When applying an empirical length to carbon regression for larvaceans (Jaspers et al. 2009), carbon biomass is underestimated by 46% when shrinkage effects due to Lugol solution are ignored. We therefore suggest that shrinkage should be carefully addressed and different rates in body size reduction be applied as suggested in the common methodology for ciliates (Putt and Stoecker 1989, Ohman and Snyder 1991, Jerome et al. 1993, Stoecker et al. 1994) and flagellates (Borsheim and Bratbak 1987, Choi and Stoecker 1989, Menden-Deuer et al. 2001).

Compared to Lugol solution, shrinkage rates due to different concentrations of aldehyde-based solutions show a lower, but significant, shrinkage of 10% to 13% for several larvacean species (Deibel 1988, Landry et al. 1994, Scheinberg et al. 2005). Additional studies reported body length reduction of three different larvacean species due to the use of 10% formalin of 3.4% after 4 months of preservation (Hopcroft and Roff 1998), and 11% due to a 5% solution for *Oikopleura vanhoeffeni* (Deibel, unpublished data from Deibel 1986). A large range of shrinkage rates has been published by the use of 4% formalin, which led to a trunk length reduction of 30.6% for *Stegasoma magnum* and 6% for *Oikopleura dioica* after several months of preservation (Alldredge 1981). This indicates that irrespective of fixative and preservation techniques, shrinkage effects for zooplankton can be substantial and should be considered. Further, despite their widespread use, the liability of aldehyde-based preservatives is the demonstrated health risk and the cost associated with proper handling facilities and waste disposal (Black and Dodson 2003, Wetzel et al. 2005), which have to be taken into account.

Our results indicate that the shrinkage effect occurs immediately after Lugol addition, as indicated by the larvacean trunk measurements 2 min after treatment. This has also been shown by Montagnes et al. (1994) for acidified Lugol solution on seven different phytoplankton groups for concentrations between 1% and 10% and measurements immediately after addition of Lugol solution for up to 3 months after treatment. This contradicts the previous experiments by Ohman and Snyder (1991), who found that acidified Lugol solution led to an immediate swelling response before shrinkage effects started 2 h later, and maximum reduction in cell size was reached 24 h after treatment. In their experiments with ciliates, the initial swelling phase was not observed for neutral Lugol solution. After the immediate shrinkage and maximum body size reduction 24–36 h after treatment, no further reduction in size seems to occur within the next 1 to 3 months (Ohman and Snyder 1991, Montagnes et al. 1994, this study).

In conclusion, acidified Lugol solution has a significant shrinkage effect on body sizes of larvaceans and copepods. The experiment analyzing the effect 2 min after treatment shows an immediate response. Therefore, we assume that the effect of acidified Lugol solution on body size for both investigation groups happens immediately upon treatment as a physiological osmotic effect. Body length to carbon regressions should consider shrinkage effects for appropriate biomass estimation.

Comments and recommendations

Our study shows that mesozooplankton organisms shrink due to the use of acidified Lugol solution, in both gelatinous and chitineous plankton groups. Compared to aldehyde-based

preservatives, Lugol solution has a stronger shrinkage effect. Acidified Lugol solution has the advantage that it is less toxic than classic aldehyde-based preservatives normally used for mesozooplankton samples. Recent studies concerning copepod nauplii experiments and quantifications use acid Lugol solution as fixative and for preservation purposes (e.g., Arendt et al. 2005, Koski 2007, Safi et al. 2007, Köster et al. 2008). Copepod nauplii fall within the same size range as ciliates and are therefore analyzed with the same methodology by settling chamber counts (Safi et al. 2007). Besides that, acid Lugol solution is preferred as fixative and for preservation purposes for zooplankton feeding experiments concerning diets with ^{14}C-labeled algae because Lugol shows the lowest loss rates (Holtby and Knoechel 1981).

However, Lugol has the disadvantage of dyeing the organic material dark brown or red (dependent on the concentration). Thereby organic matter can be easily detected but morphological details are sometimes not easily discriminated even though cilia have been shown to be better preserved in Lugol, with a preference of acidified over neutral and basic Lugol solutions (Harris et al. 2000). Samples can be bleached with bright light or addition of a few drops of 0.25 M sodium thiosulfate (Borsheim and Bratbak 1987). It should be noted that iodine-based solutions deteriorate in light and are absorbed by plastic bottles. Long-term preservation should be conducted in glass bottles, stored in the dark, and the samples checked regularly.

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